

206440US0CONT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Vitaly Arkadievich LIVSHITS et al : ATTN: APPLICATION BRANCH

SERIAL NO. NEW U.S. APPLICATION :

FILED: HEREWITH :

FOR: DNA CODING FOR PROTEIN WHICH CONFERS
ON BACTERIUM ESCHERICHIA COLI
RESISTANCE TO L-HOMOSERINE, AND METHOD
PRODUCING L-AMINO ACIDS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 5, beginning at line 3, please delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be referred to as "rhtB gene", a protein coded by the rhtB gene may be referred to as "RhtB protein", an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of making a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity", and a structural gene encoding the RhtB protein in the rhtB gene may be referred to as "rhtB structural gene". The term "enhancing the Rh activity" means imparting resistance to homoserine to a bacterium or enhancing the resistance by means of increasing the number of molecules of the RhtB protein, increasing a specific activity of the RhtB protein, or

09847391-050304
FOEC0950-2624860

desensitizing negative regulation against the expression or the activity of the RhtB protein or the like. The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild type strain thereof can not grow, usually at 10 mg/ml. The ability to produce an amino acid means a property that a bacterium produces and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

Page 6, beginning at line 4, please delete the paragraph and replace it with the following paragraph:

According to the present invention, resistance to homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. A bacterium belonging to the genus *Escherichia*, which has increased resistance homoserine and an ability to produce an amino acid, accumulates an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine or L-threonine in a medium with a high yield.

Page 6, beginning at line 15, please delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may code for a protein having the Rh activity and having an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing. Specifically, the DNA of the present invention may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing.

Page 10, beginning at line 2, please delete the paragraph and replace it with the following paragraph:

The DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by expressing a DNA subjected to in vitro mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine, and selecting the DNA which increases the resistance. Also, it is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants, and therefore the DNA, which codes for substantially the same protein, can be obtained from L-homoserine resistant species, strains, mutants and variants belonging to the genus *Escherichia*. Specifically, the DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus *Escherichia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherichia*. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

Page 17, beginning at line 14, please delete the paragraph and replace it with the following paragraph:

An amino acid can be efficiently produced by cultivating the bacterium in which the Rh activity is enhanced by amplifying a copy number of the rhtB gene as described above,

and which has an ability to produce the amino acid, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

Page 24, beginning at line 8, please delete the paragraph and replace it with the following paragraph:

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/1 kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 2 and containing 50 mg/1 kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 40 hours with a rotary shaker. After the cultivation, accumulated amounts of alanine, valine and isoleucine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

Page 25, beginning at line 11, please delete the paragraph and replace it with the following paragraph:

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/1 kanamycin and 100 mg/1 streptomycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example 2 and containing 50 mg/1 kanamycin and 100 mg/1 streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

Page 27, beginning at line 11, please delete the paragraph and replace it with the following paragraph:

Then the susceptibility of the thus obtained *E. coli* strain N99 *rhtB*::cat, of the initial strain N99 (*rhtB*⁺) and of its derivative transformed with pRhtB plasmid, N99/pRhtB, to some amino acids and amino acid analogues was tested. overnight cultures of the strains grown in M9 minimal medium at 37°C with a rotary shaker (10⁹ cfu/ml) were diluted 1:100 and grown for 5 hours under the same conditions. Then the log phase cultures thus obtained were diluted and about 10⁴ of alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. The minimum inhibitory concentration (MIC) of these compounds were examined after 40-46 h cultivation. The results are shown in Table 4.

Please delete the original Sequence Listing on pages 29-31.

Page 34, (Abstract), after the last line, beginning on a new page, please insert the attached substitute Sequence Listing.

IN THE CLAIMS

Please cancel Claims 1-7.

Please add new Claims 8-11.

8. A method for producing an amino acid selected from the group consisting of L-threonine, L-homoserine, L-alanine, L-isoleucine and L-valine, comprising the steps of :

cultivating a bacterium which has an ability to produce and accumulate the amino acid, in a culture medium, and

recovering the amino acid from the medium,

wherein said bacterium is a bacterium belonging to the genus *Escherichia*, wherein L-homoserine resistance of said bacterium is enhanced by amplifying a copy number of a DNA

in cell of said bacterium, said DNA coding for a protein as defined in the following (A) or (B):

(A) a protein which comprises an amino acid sequence shown in SEQ ID NO:2;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acid in the amino acid sequence shown in SEQ ID NO: 2, and which has an activity of making a bacterium having the protein L-homoserine-resistant.

9. The method according to Claim 8, wherein said amino acid is at least one selected from the group consisting of L-alanine, L-isoleucine, and L-valine.

10. The method according to Claim 8, wherein said DNA is carried on a multicopy vector in said bacterium.

11. The method according to Claim 8, wherein said DNA is carried on a transposon in said bacterium.

REMARKS

This is a Continuation Application of Serial No. 09/396,357 filed September 15, 1999. Claims 8-11 are active in the case.

The paper copy of the substitute Sequence Listing in this application is identical to the last-filed computer readable Sequence Listing filed in application 09/396,357 filed September 15, 1999. In accordance with 37 CFR § 1.821 (e), please use the last-filed computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the instant application. A paper copy of the substitute Sequence Listing is attached herewith.

Applicants submit that the application is now ready for examination on the merits

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
Attorney of Record
Registration No. 24,618



22850

Daniel J. Pereira, Ph.D.
Registration No. 45,518

(703) 413-3000
Fax #: (703) 413-2220

09847392 05001
T0E050" 26E24860

Marked-Up Copy

Serial No.: New U.S. Application

Amendment Filed: May 3, 2001

IN THE SPECIFICATION

Page 5, beginning at line 3, please delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be referred to as "rhtB gene", a protein coded by the rhtB gene may be referred to as "RhtB protein", an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of making a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as "Rh activity", and a structural gene encoding the RhtB protein in the rhtB gene may be referred to as "rhtB structural gene". The term "enhancing the Rh activity" means imparting resistance to homoserine to a bacterium or [enhance] enhancing the resistance by means of increasing the number of molecules of the RhtB protein, increasing a specific activity of the RhtB protein, or desensitizing negative regulation against the expression or the activity of the RhtB protein or the like. The terms "DNA coding for a protein" mean a DNA of which one of strands codes for the protein when the DNA is double-stranded. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild type strain thereof can not grow, usually at 10 mg/ml. The ability to produce an amino acid means a property that a bacterium produces and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

Page 6, beginning at line 4, please delete the paragraph and replace it with the following paragraph:

According to the present invention, resistance to homoserine of a high concentration can be imparted to a bacterium belonging to the genus *Escherichia*. A bacterium belonging to the genus *Escherichia*, which has increased resistance homoserine and an ability to [accumulate] produce an amino acid, accumulates an amino acid, especially, L-homoserine, L-alanine, L-isoleucine, L-valine or L-threonine in a medium with a high yield.

Page 6, beginning at line 15, please delete the paragraph and replace it with the following paragraph:

The DNA of the present invention [coding] may code for a protein having the Rh activity and having an amino acid sequence shown in SEQ ID NO: 2 in Sequence Listing. Specifically, the DNA of the present invention may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing.

Page 10, beginning at line 2, please delete the paragraph and replace it with the following paragraph:

The DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by expressing a DNA subjected to in vitro mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine, and selecting the DNA which [increase] increases the resistance. Also, it is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants, and therefore the DNA, which codes for substantially the same protein, can be obtained from L-homoserine resistant species, strains, mutants and variants belonging to the genus *Escherichia*. Specifically, the DNA, which

codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus *Escherichia* which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus *Escherichia*. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

Page 17, beginning at line 14, please delete the paragraph and replace it with the following paragraph:

An amino acid can be efficiently produced by cultivating the bacterium in which the Rh activity is enhanced by amplifying a copy number of the *rhtB* gene as described above, and which has an ability to produce the amino acid, in a culture medium, [producing and accumulating] to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-alanine, L-isoleucine, L-valine and L-threonine.

Page 24, beginning at line 8, please delete the paragraph and replace it with the following paragraph:

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated

into 3 ml of the fermentation medium described in Example [3] 2 and containing 50 mg/1 kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 40 hours with a rotary shaker. After the cultivation, accumulated amounts of alanine, valine and isoleucine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

Page 25, beginning at line 11, please delete the paragraph and replace it with the following paragraph:

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/1 kanamycin and 100 mg/1 streptomycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium described in Example [3] 2 and containing 50 mg/1 kanamycin and 100 mg/1 streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an absorbance at 560 nm of the medium were determined by known methods.

Page 27, beginning at line 11, please delete the paragraph and replace it with the following paragraph:

Then the susceptibility of the thus obtained *E. coli* strain N99 *rhtB*::cat, of the initial strain N99 [*(rhtB⁻)*] (*(rhtB⁺)*) and of its derivative transformed with pRhtB plasmid, N99/pRhtB, to some amino acids and amino acid analogues was tested. overnight cultures of the strains grown in M9 minimal medium at 37°C with a rotary shaker (10^9 cfu/ml) were diluted 1:100 and grown for 5 hours under the same conditions. Then the log phase cultures thus obtained were diluted and about 10^4 of alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. The minimum

inhibitory concentration (MIC) of these compounds were examined after 40-46 h cultivation.

The results are shown in Table 4.

IN THE CLAIMS

Claims 1-7 (cancelled).

Claims 8-11 (New).